1 Gene regulation is commonly selected 2 for high plasticity and low noise

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8 Abstract

9 Bacteria often respond to dynamically changing environments by regulating gene expression.
10 Despite this regulation being critically important for growth and survival, little is known about
11 how selection shapes gene regulation in natural populations. To better understand the role
12 natural selection plays in shaping bacterial gene regulation, here we compare differences in the
13 regulatory behaviour of naturally segregating promoter variants from *Escherichia coli* (which
14 have been subject to natural selection) to randomly mutated promoter variants (which have
15 never been exposed to natural selection). We quantify gene expression phenotypes (expression
16 level, plasticity, and noise) for hundreds of promoter variants across multiple environments, and
17 show that segregating promoter variants are enriched for mutations with minimal effects on
18 expression level. In many promoters, we infer that there is strong selection to maintain high
19 levels of plasticity, and direct selection to decrease or increase cell-to-cell variability in
20 expression. Finally, taking an integrated view, we show that across all phenotypes combined,
21 segregating promoter variants are far more phenotypically similar than would be expected given

22 their genetic divergence. This is the consequence of both stabilizing and directional selection
 23 acting on individual phenotypes to minimize differences among segregating variants. Taken
 24 together, these results expand our knowledge of how gene regulation is affected by natural
 25 selection and highlight the power of comparing naturally segregating polymorphisms to *de novo*
 26 random mutations to quantify the action of selection.

27 Main

28 Gene regulation plays a critical role in determining the physiology and behaviour of unicellular
 29 organisms. As such, regulation of gene expression is subject to natural selection. There are at
 30 least three aspects of gene regulation that are under selection and which affect transcription
 31 and downstream phenotypes. The first of these is expression level (the amount of protein
 32 produced from a gene). There is experimental evidence that expression levels of some genes
 33 are close to optimal (Hawkins et al., 2020; Keren et al., 2016), and furthermore, can rapidly be
 34 tuned to optimise fitness within an environment (Dekel & Alon, 2005). However, only a few
 35 studies have systematically tested the range of expression levels that have no effect on fitness,
 36 and whether this range differs between genes (Keren et al., 2016).

37 In contrast to the understudied role of natural selection on gene expression level, a considerable
 38 amount is known about the molecular mechanisms controlling expression level, especially in
 39 model organisms. For example, for the vast majority of genes and operons in the bacterium *E.*
 40 *coli*, the specific sigma factors, transcription factors, and their binding sites are well documented
 41 (de Boer et al., 2020; Ireland et al., 2020). In many cases, the specific biochemical interactions
 42 that affect expression level have been thoroughly investigated (Brewster et al., 2012, 2014;
 43 Gertz et al., 2009).

44 A secondary aspect of expression level is plasticity - changes in gene expression that occur
 45 when cells encounter different environmental signals. There are several aspects of phenotypic
 46 plasticity that are subject to selection; among these are the level of the transcriptional response,
 47 the speed of the response, and the sensitivity of the response (Maeda & Sano, 2006; Mangan et
 48 al., 2006; Rosenfeld et al., 2002). Although these plastic responses are well characterised, as
 49 with gene expression levels, little is known about whether stabilizing, directional, or diversifying
 50 selection is most influential in shaping phenotypic plasticity. One exception to this is the work
 51 that has been done to characterise the action of selection on the plasticity of the TDH3 promoter

52 in yeast (Duveau et al., 2017). However, there is limited knowledge about phenotypic plasticity
 53 in bacteria and even in eukaryotes only a limited number of promoters have been studied
 54 (Duveau et al., 2017; Hill et al., 2021; López-Maury et al., 2008).

55 As with expression level, an abundance of research has been done on the molecular
 56 mechanisms underlying phenotypic plasticity. As noted above, many of the transcription factors
 57 that cause changes in transcription are well characterised. In addition, the specific topologies of
 58 regulatory networks that promote specific types of responses have been extensively studied
 59 (Basu et al., 2004; Becskei & Serrano, 2000; Eisen et al., 1970; Kalir et al., 2005; Mangan &
 60 Alon, 2003; Novick & Weiner, 1957; Shen-Orr et al., 2002; Smits et al., 2006), including how
 61 different topologies influence the phenotypic effects of mutations (Madan Babu et al., 2006;
 62 Mayo et al., 2006; Metzger & Wittkopp, 2019; Schaerli et al., 2018).

63 In contrast to expression level or plasticity, a large number of studies have quantified how
 64 selection acts on expression noise - the amount by which individual isogenic cells differ in
 65 expression level. For example, several studies have compared the noise exhibited by different
 66 promoters within the same organism to understand whether specific promoters have been
 67 selected such that they confer high or low levels of noise (Bar-Even et al., 2006; Elowitz et al.,
 68 2002; Rossi et al., 2019; Silander et al., 2012; Süel et al., 2007; Taniguchi et al., 2010; Wolf et
 69 al., 2015). There is evidence that promoters of essential genes exhibit low levels of noise, and
 70 this has been observed for both bacteria and eukaryotes (Metzger & Wittkopp, 2019; Silander et
 71 al., 2012; Urchueguía et al., 2019). In addition, modeling has shown that there can be a
 72 selective advantage for genes with high plasticity to exhibit high noise levels if the regulation of
 73 gene expression is not precise (Wolf et al., 2015). High noise levels from some promoters can
 74 lead to phenotypically distinct populations of isogenic cells coexisting in the same environment
 75 (Govers et al., 2017; Kotte et al., 2014; Ronin et al., 2017). This can be selectively
 76 advantageous as a bet-hedging phenomenon, which is sometimes beneficial in highly variable
 77 or unpredictable environments (Acar et al., 2008; Veening et al., 2008). One of the most studied

78 examples of bet-hedging is bacterial persistence, which is known to result in a small
79 subpopulation of cells which tolerate antibiotic exposure without acquisition of antibiotic
80 resistance genes (Lewis, 2007).

81 The specific molecular mechanisms that can affect noise levels are also well-studied. For
82 example, promoters in yeast with TATA boxes are generally more noisy than those lacking these
83 sequences. Random mutations in TATA boxes have been found to decrease both expression
84 noise and phenotypic plasticity (Hornung et al., 2012; Richard & Yvert, 2014). In contrast, yeast
85 promoters lacking TATA boxes have been found to be mutationally robust (Hornung et al.,
86 2012). In *E. coli*, specific transcription factors are associated with promoters conferring high or
87 low levels of noise (Silander et al., 2012). Finally, there are general features of gene regulation
88 that promote or dampen noise. For example, genes that have low rates of transcription and high
89 rates of translation will generally have higher noise levels (Jones et al., 2014).

90 Here, we investigate how selection acts on different gene expression phenotypes by selecting
91 ten *E. coli* promoters which exhibit different levels of segregating variation. We quantify
92 expression phenotypes (expression level, plasticity, and noise) from these segregating promoter
93 variants in three environments specific for each promoter. Finally, to elucidate whether
94 stabilizing, directional, or diversifying selection has shaped gene regulation, we compare the
95 expression from the segregating promoter variants, which have been exposed to natural
96 selection, to a set of variants generated through random mutation, and which have never been
97 exposed to selection.

98 Results

99 Sequence variation in segregating promoters

100 To characterise the relationship between genetic variation, phenotypic variation, and selection
 101 on gene regulation, we first quantified genetic variation in intergenic regions (IGRs) and open
 102 reading frames (ORFs) from 135 environmental isolates of *E. coli* (Ishii et al., 2006) that span
 103 the genetic diversity of *E. coli* (Sakoparnig et al., 2021). We assumed that a substantial portion
 104 of gene regulatory phenotypes depend on the sequence of these IGRs. We used annotations in
 105 RegulonDB to identify 605 of IGRs with known transcription start sites in *E. coli* MG1655, and
 106 which are controlled by the sigma factor σ^{70} (Santos-Zavaleta et al., 2019). We also included the
 107 corresponding downstream annotated ORFs (Blattner et al., 1997). We then identified and
 108 aligned homologous regions from the 135 environmental isolates of *E. coli* (Breckell & Silander,
 109 2020; Sakoparnig et al., 2021). To increase the likelihood of capturing homologous IGRs, we
 110 included 100 base pairs (bp) of the upstream and downstream ORFs (see **Methods**). Using
 111 these alignments, we calculated the proportion of segregating sites (PSS) and average pairwise
 112 identity (API) for each IGR (excluding the ORF portions of the alignments), as well as for the
 113 downstream ORFs (**Fig. 1a** and **Supplementary Figure 1a**). We found that the PSS in IGRs
 114 varied by more than an order of magnitude, from 0.369 (one indel and 104 SNPs across 282 bp
 115 for the IGR acting as a bidirectional promoter for the transcriptional dual regulator *meIR* and
 116 α -galactosidase *meIA*) to 0.007 (the IGR upstream of 30S ribosomal subunit protein *rpsM*, one
 117 SNP in 146 bp). We also observed perfect conservation for the IGRs upstream of the zinc
 118 metalloprotease *ftsH* (99 bp in length), the octaprenyltransferase *menA* (66 bp), the PF03966
 119 family protein *ycaR* (51 bp), and the IGR that acts as as a bidirectional promoter for the
 120 transcriptional dual regulators *soxR* and *soxS* (85 bp). The API of IGRs ranged from 88.14%

121 (one indel and 27 SNPs across 75 bp for the IGR upstream of bacterioferritin *bfr*) to 100% (*ftsH*,
 122 *menA*, *ycaR*, *soxR*, and *soxS*).

Figure 1: Polymorphisms in intergenic regions (IGRs) and open reading frames (ORFs) across 135 environmental isolates of *E. coli* and MG1655. a) The proportion of segregating sites (PSS) for IGRs and downstream ORFs varies by more than an order of magnitude. Each blue dot indicates PSS for an IGR-ORF pair when the IGR contains a transcriptional start site for the ORF. In red are IGRs that we selected for further study due to their different levels of sequence variation (*aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA*; see **Table 1**). **b)** The proportion of segregating sites for IGRs differs little among different functional groups, as classified by the downstream ORF. CH denotes conserved-hypothetical. Unclassified, ORFs, and CH-ORFs all represent groups of ORFs with very limited information on function (Serres & Riley, 2000). Numbers next to each function group represent the p-value obtained by performing the Wilcoxon rank-sum test to test for differences in PSS in each group from all other PSS values for IGRs outside of the group. Bold indicates significant p-values after the Bonferroni correction for multiple comparisons among functional groups. The black lines indicate the median PSS values of each functional group.

123 In order to understand whether selection had acted in a general manner to affect the levels of
 124 polymorphism, we tested whether ORFs in certain functional categories had upstream IGRs
 125 with high or low sequence variation. We found that genes involved in information transfer were
 126 enriched for IGRs with lower levels of polymorphism (median PSS 0.084 compared to 0.104 for
 127 all other IGRs, $p = 0.005$; median API 98.87% compared to 98.51% for all other IGRs, $p =$
 128 0.004; **Fig. 1b, Supplementary Figure 1b**). This lower level of variation may be due to stronger
 129 stabilising selection on the regulation of ORFs involved in information transfer. The correlation
 130 between PSS and inverted API values for IGRs is high (Spearman's $\rho = 0.946$, $p = 4.3e-211$)
 131 making the two measures interchangeable. PSS and API data for all IGRs and ORFs present in
 132 at least 130 out of 135 environmental strains are in **Supplementary File 1**.

133 A system to quantify the effects of mutations on promoter phenotypes

134 To investigate in more detail the selective mechanisms responsible for conferring different levels
 135 of genetic variation in IGRs, we selected ten IGRs varying widely in PSS and API (**Table 1, Fig.**
 136 **1a, Supplementary Figure 1a**; red points). We selected these based solely on genetic variation
 137 and for which literature data suggested phenotypic plasticity in certain environments.
 138 For each of these ten IGRs, we cloned the segregating variants upstream of green fluorescent
 139 protein (GFP) on a low copy-number plasmid (**Fig. 2a, 2b and 2d**). In all cases, the cloned
 140 regions included the IGRs together with 40 - 110 bp of coding sequences flanking the IGRs to
 141 include regulatory sequences that might be present outside the IGRs. We PCR amplified the
 142 promoter region from a DNA pool of the segregating variants. Accordingly, across all ten
 143 promoters, we obtained 75% (134 out of 179) of the segregating variants (**Table 1**). We
 144 transformed all the resulting plasmids into *E. coli* MG1655, such that they were all present in a
 145 single isogenic background. We expect that if promoter phenotypes are more similar in their
 146 respective native genetic backgrounds, then this change in genetic background will only

147 increase phenotypic differences. Thus, any conclusions we make on the similarity in the
148 behaviour of segregating variants are conservative.

Promoter	PSS	API	Number of segregating variants		Function(s) of downstream ORF	Environment abbreviation*
			cloned	total		
<i>aldA</i>	0.20	96.70%	25	34	metabolism	Glu Fuc Gly
<i>yhjX</i>	0.20	95.50%	16	24	cell structure, transport	Glu Pyr Pyr2
<i>lacZ</i>	0.18	96.19%	20	27	metabolism	Glu Gal Lac
<i>aceB</i>	0.14	97.87%	18	27	metabolism	Glu Pyr Mal
<i>mtr</i>	0.13	97.83%	19	26	metabolism, cell structure, transport	Glu Try Phe
<i>cdd</i>	0.09	98.78%	12	19	metabolism	Glu Cyt Amp
<i>dctA</i>	0.07	99.52%	7	8	metabolism, cell structure, transport, regulation	Glu Pyr Mal
<i>ptsG</i>	0.06	99.16%	10	15	metabolism, cell structure, transport, regulation, information transfer	Glu Gly Man
<i>purA</i>	0.04	99.57%	4	7	metabolism	Glu Gly Ara
<i>tpiA</i>	0.02	99.76%	3	5	metabolism	Glu Gly Ara

Table 1: Characteristics of promoters selected for phenotypic assays. PSS - proportion of segregating sites in promoters, API - average pairwise identity in promoters. The functional groups of downstream genes were obtained using MultiFun (Serres & Riley, 2000).

* For the full description of the assay environments see **Table 2**.

149 We refer to these IGRs and proximal regions as promoters. We note that while differences
 150 between promoters in the expression phenotypes they confer are necessarily due to differences
 151 in the DNA sequence, it is not necessarily due to differences solely in transcription, but to any
 152 differences in the levels of transcriptional or translational regulation. Secondly, although this is a
 153 plasmid-based system, we have shown previously that expression phenotypes are
 154 well-correlated with those observed when these constructs are assayed in a chromosomal
 155 context (Silander et al., 2012).

156 Inclusion of the regions from the flanking ORFs had little effect on the relative genetic
 157 variabilities we observed (Spearman's correlation for the regions inclusive and exclusive of the
 158 flanking ORFs, $R = 0.918$ for PSS, $p = 1.09\text{e-}180$; $R = 0.885$ for API, $p = 1.05\text{e-}149$;
 159 **Supplementary Figure 1c and 1d**).

160 For each promoter we then identified specific environments in which it exhibited different
 161 expression levels. As it is well-established that glucose is the preferred carbon source for *E. coli*
 162 (Monod, 1949), we included this as an environment for all promoters, assaying the activity
 163 during exponential growth in M9 minimal salts media with 0.4% glucose (Glu, **Table 1** and **2**).

164 We used information from the literature on the behaviour of the MG1655 promoter variants to
 165 identify additional environments in which we expected a promoter to exhibit different expression
 166 levels. We confirmed this behaviour using flow cytometry. In this way, for each promoter, we
 167 identified two additional environments in which we observed differential expression (**Table 1** and
 168 **2, Figure 2e and 2f**). We found that all segregating variants behaved consistently in all the
 169 environments that we tested. In the case of *yhjX*, we were not able to obtain expression levels
 170 higher than cellular background fluorescence in any environment that we tested except pyruvic
 171 acid. Nevertheless, we found that the promoter was very responsive to the concentration of
 172 pyruvic acid. We thus used two different concentrations of pyruvic acid as a carbon source to
 173 achieve differential levels of expression.

Figure 2: Experimental design to assay the effects of segregating and random mutations on gene expression. **a)** We isolated ten promoters (*aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA*) originating from MG1655. **b)** We then PCR amplified variants of these ten promoters segregating among environmental *E. coli* isolates from DNA pools. The average number of mutation across all segregating variants (as compared to MG1655) is 7.2 (ranging from 1 to 12.7 for individual promoters) **c)** We also performed PCR random mutagenesis using each of the ten MG1655 promoters with a target mutation rate of 1.5 mutations per promoter sequence. **d)** We cloned the resulting PCR amplicons (both segregating and random) into the pUA66 vector upstream of GFPmut2 (Zaslaver et al., 2006). We Sanger sequenced all the promoter variants to confirm the presence and location of mutations. From mutagenesis only the variants containing 1 to 3 SNPs were used for further phenotypic assays. **e)** We then cultured each of these individual promoter variants (1000 in total) in three different environments in triplicates, and **f)** quantified the modal population expression and coefficient of variation levels using flow cytometry.

174 Relationship between segregating genetic variation and phenotypic

175 variation is correlated

176 We hypothesized that the differences we observed in the genetic variation present for different
 177 promoters might be a consequence of differences in the range of optimal expression levels. If
 178 the optimal range was very narrow, then all variants of that promoter should exhibit similar
 179 expression levels, and new mutations that affect expression should be filtered by selection,
 180 decreasing genetic diversity. In contrast, if there were a wide range of optimal expression levels

181 within an environment, then variants can differ in expression levels, selection would act weakly
 182 against new mutations, and segregating genetic diversity would be high. Under this hypothesis,
 183 there should be a positive correlation between genetic variation and phenotypic variation. A
 184 second possibility is that segregating variants have very little effect on phenotype and are
 185 selectively neutral. In this case we would expect no correlation between genetic variation and
 186 phenotypic variation.

187 To test for such a correlation, for each promoter, we measured the modal population expression
 188 level from each segregating variant in each environment. In all cases, we measured the modal
 189 population expression from three full biological replicates (**Methods**). As a measure of
 190 phenotypic variation, we calculated the standard deviation in log expression level across all
 191 segregating variants of each promoter. We found that promoters with more segregating genetic
 192 variation tended to have larger phenotypic variation in expression (**Fig. 3a** and **3b**,
 193 **Supplementary Figure 2a** and **2b**; Spearman's $\rho = 0.666$, $p = 5.8e-05$). While this evidence
 194 does not conclusively show that the lower level of genetic variation in some promoters is due to
 195 the increased strength of stabilising selection, it shows that our experimental system is capable
 196 of detecting the effects of segregating mutations on expression phenotype.

Figure 3: Segregating genetic variation in promoters correlates with phenotypic variation in expression levels. a) and b) Standard deviations in modal expression levels from segregating variants are correlated with the genetic variation of the promoter (IGR with 100 bp flanking regions). Panel **a** shows the correlation with PSS and panel **b** shows the correlation with the total number of segregating promoter variants. For each promoter, the standard deviation of modal population expression was measured in three environments (three dots per promoter, **Table 2**). The rho and p-values were calculated using Spearman's correlation test.

197 Effects on expression level are environment-dependent

198 Segregating promoter variants have evolved under the pressure of natural selection. Despite
 199 this, some segregating variants differ by up to 27 SNPs from the MG1655 variant (the putative
 200 transporter *yhjX*), and we have found that these mutations have detectable phenotypic effects -
 201 promoters with larger levels of sequence variation tend to have a larger range in modal
 202 expression levels (**Fig. 3a** and **3b**). To investigate how selection has affected the level of
 203 segregating variation, we created a set of promoter variants with mutations that had not been
 204 subject to selection. To do this we used PCR mutagenesis on the MG1655 promoter variant, as
 205 a representative of segregating versions of each of the ten promoters. We cloned these
 206 upstream of GFPmut2 gene on a plasmid and transformed them into MG1655, creating a set of

207 promoter variants exactly analogous to the segregating variants, but containing mutations that
 208 had never been subject to the action of selection (**Fig. 2c** and **2d**).

209 To identify the specific mutations that each of these new promoters contained, we sequenced
 210 192 clones for each of the ten promoters (1,920 clones in total; see **Methods**). We filtered out
 211 all duplicated variants and any containing none or more than three *de novo* random mutations.
 212 Next, for nine of the ten promoters, we selected 93 randomly mutated variants to produce a
 213 well-characterised library of promoter variants containing mutations that have never
 214 experienced the action of natural selection. For the tenth promoter, *lacZ*, we obtained only 29
 215 random variants (see **Methods**). Given the small sample size, selective effects will be more
 216 difficult to detect, as statistical significance will generally require larger effect sizes.

217 Using these libraries, we first characterised the effects of the randomly introduced SNPs on
 218 expression level, considering only promoters with a single SNP (**Fig. 4a**). As expected
 219 (Belliveau et al., 2018; Kinney & McCandlish, 2019), we found that mutations with the largest
 220 effects on expression in all environments and promoters were present in the regions required for
 221 σ^{70} binding (-35 or -10 elements) or annotated transcription factor (TF) binding sites ($p =$
 222 $2.27\text{e-}09$ for larger fold-changes in expression for SNPs within vs. outside of σ^{70} and TF binding
 223 sites, Wilcoxon rank-sum test). However, these data also showed that both the direction (i.e. an
 224 increase or decrease in expression level) and size of the mutational effects were dependent on
 225 the growth environment. In some promoter-environment combinations almost half of the
 226 mutations resulted in increased expression, while in a different environment these same
 227 mutations decreased expression. For example, comparing the effects on expression level in
 228 pyruvic acid vs. L-malic acid, 44.2% of all single mutations (both inside and outside TF binding
 229 sites) in *aceB* and 31.11% of all single mutations in *dctA* had opposing effects (**Fig. 4b**). In other
 230 cases, the effect sizes of the mutations were environment-dependent (e.g. compare *aldA* in
 231 glucose vs. fucose or *yhjX* in glucose vs. pyruvic acid; **Fig. 4a**).

232 We note that the MG1655 *mtr* variant is expressed at lower levels, which is likely caused by a
 233 non-synonymous mutation in GFP (**Supplementary Note**). The changes in expression in **Fig. 4**
 234 for the *mtr* promoter, most notably for glucose with phenylalanine (Glucose + Phe) thus do not
 235 accurately represent the magnitude of the changes to native MG1655 promoter variant.
 236 All together, these results emphasize that in order to gain general insights into the effects of
 237 mutations on gene expression phenotypes, it is necessary to measure promoter activity across
 238 different growth environments. Not only did we observe that the relative effects of random
 239 mutations on promoter activity varied across promoters, but both the size and direction of
 240 effects varied across environments (**Fig. 4b**). However, due to the action of selection, this may
 241 not be true for naturally segregating promoter variants - for example all large-effect mutations
 242 may be filtered by selection. To quantify the action of selection, we next investigated differences
 243 between the phenotypic effects of naturally segregating and *de novo* random mutations.

Figure 4: The effects of random mutations on expression level are promoter and environment dependent. **a)** Changes in expression from MG1655 promoter variants due to single random mutations for all environments. For each promoter, the x-axes indicate the position relative to the start codon of the gene downstream of each promoter. The horizontal black line indicates the expression level of the MG1655 promoter variant in that environment. The vertical black lines show the direction and size of the change in expression level when the MG1655 promoter variant is mutated at that specific position. While in certain environments some mutations cause no change in expression phenotype in other environments, the same mutations can cause up to 10-fold changes in expression. The results shown here are only from random promoter variants containing a single mutation relative to the MG1655 promoter variant. Note that for *mtr*, the MG1655 variant has a mutation in the downstream GFP, causing lower fluorescence (**Supplementary Note**), and thus the random mutations exhibit larger effects when the promoter is activated. The brown stripes result from overlapping repressor and activator TF binding sites. **b)** Comparison of differences in modal expression from random variants with single SNP relative to the MG1655 variant for *aceB* and *dctA* promoters. Differences are always compared between a pair of environments. Blue dotted lines indicate a null change in expression as compared to the MG1655 variant. These lines also divide each plot into four quadrants. Data points in the bottom left and top right quadrants show changes in expression in the same direction (decrease or increase) in both environments. Variants clustering in top left and bottom right quadrants exhibit changes in the opposite directions in the two environments (increase in one environment and decrease in the other). The numbers in each quadrant represent the proportion of variants falling into that quadrant. Each data point in **a** and **b** is a result of three full biological replicates (**Methods**).

244 Segregating polymorphisms are enriched for mutations with small effects 245 on expression level

246 We first compared the expression levels of each of the 93 random variants per promoter (29 in
247 the case of *lacZ*) to the expression levels of segregating promoter variants. Specifically, for each
248 promoter and each environment, we tested whether expression levels among random mutants
249 varied more than expression levels among segregating promoter variants. In all cases in which
250 we observed significant differences, we found that random promoter variants differed more in
251 expression levels than did segregating variants (**Fig. 5**). This is despite the far larger numbers of
252 segregating mutations (on average 7.2 mutations different compared to MG1655) compared to
253 randomly generated mutations (on average 1.5 mutations different compared to MG1655). This
254 shows conclusively that segregating mutations are enriched for mutations with small effects on
255 expression levels, across a range of environments and promoters having different levels of
256 segregating diversity (**Fig. 5**).

257 In the majority of cases, the MG1655 promoter variant is representative of other segregating
 258 versions. However, we found two cases in which the MG1655 promoter variant behaved
 259 differently than all other segregating promoter variants. In the case of *aldA*, all segregating
 260 variants exhibited lower expression levels than the MG1655 variant across all conditions, except
 261 for a single variant in fucose (**Fig. 5**). In addition, the expression levels of promoter variants
 262 obtained via random mutagenesis of the MG1655 variant clustered around MG1655. The
 263 increased expression in the MG1655 variant is likely due to a single SNP that changes the -35
 264 element from TGCCGT to TTCCGT, which is more similar to the canonical -35 motif (Harley &
 265 Reynolds, 1987). In addition, the MG1655 *mtr* variant was expressed at lower levels likely linked
 266 to a single SNP in the GFP gene, as mentioned above (**Supplementary Note**) and was thus
 267 excluded from the analysis.

Figure 5: Selection acts against mutations with large effects on expression levels. We calculated the modal population expression level for all segregating and random promoter variants, and tested whether segregating variants differed in expression levels more or less than random variants. If segregating promoter variants exhibit similar expression levels (i.e. they have low variance), compared to random promoter variants, then we can conclude that large-effect mutations are filtered out via selection. For each promoter, the expression level of each group of variants is plotted. Segregating variants are shown in grey, random variants in red, and the MG1655 variant in green. The expression levels during growth in each of three different environments are shown in each plot and indicated on the x-axis (**Table 2**). Here, as in the other figures, the panels are arranged in decreasing order of segregating genetic variation (PSS). As noted in **Fig. 4**, the MG1655 *mtr* promoter variant has a mutation in GFP, causing lower fluorescence, which is most apparent when the promoter is activated (**Supplementary Note**), we thus omitted it from the calculations. The numbers above each pair of segregating and random variants is the p-value obtained through the Fligner-Killeen test to test for differences in variances between the two groups. The numbers in bold indicate significant p-values after the Bonferroni correction for multiple comparisons within the promoter.

268 Segregating polymorphisms are selected to maintain high levels of 269 phenotypic plasticity

270 A key role of promoters is to control expression of downstream genes in order to provide optimal
271 levels of gene product in particular environments, i.e., phenotypic plasticity. To understand how
272 selection acts on phenotypic plasticity, we again compared the phenotypes of the library of
273 random variants to the segregating variants. We hypothesised that frequently, genes involved in
274 the metabolism of specific substrates will be selected such that their expression is highly plastic
275 (i.e. environment-dependent). We thus compared the environmental dependence of expression
276 levels for all segregating and random promoter variants in all three environments. When
277 considering two environments, highly plastic promoters should result in high expression levels in
278 one environment, and low expression in the second environment. Less plastic promoters would
279 have similar expression levels across both environments. We quantify this plasticity by
280 calculating the distance of each promoter variant from an isocline specifying identical
281 expression in both growth environments (**Fig 6a** and **Supplementary Figure 3**). This same
282 logic can be applied to three environments: promoters with similar levels in all three
283 environments are defined as having low plasticity, while strong differences in expression levels
284 in each of the three environments will be observed in highly plastic promoters. Thus to quantify
285 plasticity, we calculated the Euclidean distance of each promoter variant in three dimensions
286 (i.e. the three environments) from an isocline representing equal expression levels in all three
287 environments (**Fig. 6b**). This distance is 0 for promoter variants that are expressed at the same
288 level in all environments, and greater than zero for promoter variants that are expressed at
289 different levels, with a maximal value that is dependent on the maximum difference in
290 expression levels between any two environments.

291 As shown above (**Fig. 5**), many random mutations have large effects on expression, and we
292 observed many mutations that increased as well as decreased expression levels. However, this

293 was not true for plasticity. Only rarely did random mutations increase plasticity. For four out of
 294 the ten promoters, we found significant differences in plasticity between segregating and
 295 random variants (**Fig. 6c**; excluding *aldA* promoter results - see below), with random promoter
 296 variants exhibiting lower plasticity. Using the median plasticity of segregating promoter variants,
 297 92.47% (*aceB*), 75.27% (*mtr*), 76.34% (*cdd*), and 86.02% (*ptsG*) of the random variants
 298 exhibited lower plasticity. This strongly suggests that segregating promoter variants are under
 299 strong selection for high plasticity in these cases. Furthermore, this is almost certainly a
 300 conservative estimate of the strength of selection on plasticity, as we have assayed expression
 301 in only three environments.

302 Interestingly, when we calculated plasticity only for pairs of environments (**Supplementary**
 303 **Figure 3**) we found a case when the random variants exhibited significantly higher plasticity
 304 than the segregating variants - *dctA* in pyruvic vs. L-malic acid (76.34% of random variants have
 305 higher plasticity than is the median for segregating variants; median plasticity of 0.184 for
 306 random variants as compared to a median of 0.073 for segregating variants, $p = 0.009$; **Fig. 6d**
 307 and **6e**, Pyr:Mal). However, the difference in plasticity across all three environments was not
 308 significant ($p = 0.199$; **Fig. 6c**). This was the only case in which the data implied that there was
 309 selection for low plasticity, i.e., for similar levels of expression across different environments.

310 Finally, in the case of *aldA*, we found that the MG1655 variant exhibited considerably lower
 311 plasticity than all other *aldA* segregating variants (**Fig. 5**). The random variants have similar
 312 plasticity as MG1655, resulting in a strong difference in plasticity between the segregating and
 313 random variants. (**Fig. 6c**, *aldA* and **Supplementary Figure 3**, *aldA*). Despite this, if we
 314 compare the plasticity of the random variants to that of MG1655, we find that 67.74% exhibit
 315 lower plasticity. This suggests that even though the MG1655 variant has lower plasticity than
 316 other segregating variants, selection has still acted to filter variants that decrease plasticity.

Figure 6: Selection on plasticity. Comparison of expression levels from promoters in a combination of environments. **a)** For the *aceB* promoter in pyruvic acid and L-malic acid, the segregating variants tend to have high expression in pyruvic acid and low expression in L-malic acid, indicating high plasticity in these two dimensions. **b)** The same projection as **a** but using all three environments. The blue dotted lines in **a** and **b** indicate the isocline of equal expression levels in all environments, i.e., no phenotypic plasticity. The black arrows illustrate the plasticity for the MG1655 promoter variant (measured as the minimal distance of each point from the blue isocline, i.e., $x = y$ for **a** and $x = y = z$ for **b**). The further from this isocline a promoter variant is, the higher its phenotypic plasticity. **c)** Plasticity calculated as in **b** for all environments and all ten promoters. Segregating promoter variants often have higher levels of plasticity across the three environments. The promoters are arranged in decreasing order of segregating genetic variation (PSS). The MG1655 variant of the *mtr* promoter was omitted from calculation due to a SNP in GFP (**Supplementary Note**). **d)** Expression of all *dctA* variants in pyruvic acid and L-malic acid. In this promoter, we detected low levels of plasticity in segregating variants as compared to random variants in these two environments. **e)** Calculating the plasticity of *dctA* using pairs of environments we found that in pyruvic acid and L-malic acid segregating promoters exhibited lower plasticity ($p = 0.009$). Numbers above each pair of segregating and random variants in **c** and **e** are the p-values obtained via Wilcoxon rank-sum test to test for differences between the two groups. The numbers in bold indicate significant p-values. The horizontal black lines in **c** and **e** indicate the median plasticity values of each group.

317 Segregating polymorphisms are enriched for mutations that both increase

318 or decrease noise

319 Finally we also looked at the role of selection on noise levels among our ten promoters. Here,

320 we use the term “noise” to refer to the differences between isogenic cells in their protein

321 expression level. Promoters conferring high levels of noise result in isogenic cells having

322 different protein expression levels, while promoters conferring low noise result in almost all cells

323 having the same expression level. We note that noise is highly dependent on expression level

324 due to the stochastic production of mRNA and protein. In general, promoters that confer low

325 expression exhibit higher noise, while promoters that confer high expression exhibit lower noise.

326 Because there is strong selection on the expression level of a protein, we first calculated a

327 metric that allowed us to decouple noise from expression level. Specifically, we calculated the

328 vertical deviation from a fitted smooth spline on the log of the modal expression levels versus

329 the coefficients of variation (**Methods**). We then fitted the spline using all variants (segregating

330 and random) for a particular environment (**Supplementary Figure 4**). Thus, positive deviations

331 from the fitted spline indicate promoters with high noise, while negative values indicate
332 promoters conferring low noise (Silander et al., 2012).

333 We found that eight out of ten promoters had significantly different levels of noise (after
334 Bonferroni correction for multiple tests for each promoter) in segregating variants as compared
335 to random variants in at least one environment (**Fig. 7**). In the majority of cases, noise was
336 lower for segregating variants. As we calculated the noise metric separately for each promoter
337 and each condition, this strongly suggests that selection has acted directly to decrease noise.
338 The differences in noise are not due to differences in regulatory inputs, or to growth conditions,
339 but to subtle changes in the sequence of the promoter and the resulting phenotypes. It is
340 possible that some of these effects are mediated by changes in the binding strength of various
341 transcription factors. However, this is unlikely to be the primary factor. We would expect the
342 major effect of these changes to be expression level, and this noise metric explicitly accounts
343 for expression level.

344 However, segregating promoter variants did not exhibit lower noise in all cases. Specifically,
345 segregating *aceB* variants exhibited higher noise when grown in L-malic acid (median noise
346 deviation $7e-03$ for segregating variants compared to $-2e-03$ for random variants, $p = 0.004$).
347 Notably, in the two other environments that we tested, pyruvic acid and glucose, segregating
348 *aceB* variants exhibited lower noise. In 13 cases out of 30 we did not detect a significant
349 difference in noise between the two groups. In these cases, we expect that the differences are
350 below our limit of detection, or that random mutations are just as likely to result in increased
351 noise as they are in decreased noise. Finally, we note that we have measured noise in only a
352 very limited number of environments; it is possible that in other environments, such as those
353 with high stress, the majority of promoters would exhibit higher noise.

Figure 7: Selection on noise. Segregating promoter variants are selected such that they have low noise in most environments. Here, noise refers to the deviation from a spline fitted of modal population expression vs. the standard deviation in expression divided by the modal population expression (**Supplementary Figure 4**). **a)** Differences in noise between segregating (black) and random (red) variants. The black lines indicate the median noise value for each group. We tested for differences in noise between segregating and random variants using the Wilcoxon rank-sum test; the resulting p-values are indicated above each pair. The numbers in bold indicate significant p-values after the Bonferroni correction for multiple comparisons within the promoter. The specific environment in which the promoters were tested is indicated on the x-axis (**Table 2**). The panels are arranged in decreasing order of segregating genetic variation (PSS). The MG1655 variant of the *mtr* promoter was omitted from calculation due to a SNP in GFP (**Supplementary Note**). **b)** and **c)** show noise deviations for the *aceB* and *purA* promoters using a wider scale.

354 Segregating polymorphisms are enriched for mutations with small

355 phenotypic effects across multiple expression phenotypes

356 So far we have focused on how natural selection affects individual expression phenotypes:
357 expression level, phenotypic plasticity, and noise. However, in natural environments all these
358 aspects are simultaneously under selection. While a particular random variant may exhibit
359 behaviour comparable to the segregating variants for a single phenotype, this may not be the
360 case across all the phenotypes. To compare the behaviours of segregating and random variants
361 across all the expression phenotypes, we first made the assumption that regulatory phenotypes
362 are under stabilizing selection, and thus that the mean phenotype of the segregating promoters
363 is close to the optimum. This is clearly supported by the above results, in particular that
364 segregating mutations are enriched for mutations of small effect. Under this assumption,
365 regulatory phenotypes that deviate from this mean are less fit. Thus, we calculated the mean
366 and standard deviation of each phenotype for all segregating variants. We then used these
367 values to calculate a z-score for all variants and phenotypes. We converted these z-scores into
368 absolute values to focus only on the relative difference of the phenotype of the variant, rather
369 than the directionality. Finally, given that we have little information on which of these phenotypes
370 is under stronger selection, we weighed all phenotypes equally, and calculated the sum of
371 absolute values of the z-scores. This metric is indicative of how individual variants differ in their

372 overall phenotype set from the phenotype of the average segregating variant. We found a
 373 correlation between the z-scores of different expression levels, as well as between expression
 374 levels and plasticity. For expression levels we thus used z-scores from just one out of the three
 375 environments. This eliminated the non-independence of individual z-scores among expression
 376 levels. However, some non-independence between the z-scores of expression level and
 377 plasticity remained (**Methods**).

Figure 8: Overall selection pressure. Comparison of cumulative z-scores of all measured phenotypes (expression level, plasticity, and noise) between segregating and random variants. The numbers above each pair of segregating and random variants indicate the p-values for a Wilcoxon rank-sum test to test for differences between the two groups. The numbers in bold indicate significant p-values. The horizontal black lines indicate the median values of cumulative z-scores of each group. The promoters are arranged in decreasing order of segregating genetic variation (PSS). The MG1655 variant of the *mtr* promoter was omitted from calculation due to a SNP in GFP (**Supplementary Note**). The inset shows the full scale of cumulative z-scores on the y-axis for comparison of high values in *aceB* and *purA* promoters.

378 Using this metric, we found that in all cases except for *lacZ*, random variants exhibited
 379 significantly higher cumulative z-scores than did segregating variants (**Fig. 8**). Surprisingly,
 380 some random promoter variants (e.g. *purA*) exhibited cumulative z-scores over 100, indicating
 381 that their phenotypes were more than 100 standard deviations outside of the segregating
 382 variants. In the case of both *purA* and *tpiA*, all random variants had cumulative z-scores higher
 383 than the highest z-score of any segregating variant. These results highlight that although for
 384 individual phenotypes there are not necessarily strong consistent differences between all
 385 segregating and all random variants, considering all phenotypes together clearly illustrates that
 386 random mutations generally have much larger effects on regulatory phenotypes than do
 387 segregating mutations, and that such mutations are effectively filtered by selection.

Discussion

The effect of selection on gene expression phenotypes has not been well-studied, despite gene expression phenotypes having critical effects on cellular physiology and behaviour. To gain insight into the action of selection on gene expression, here we have compared the phenotypes of segregating variants for ten promoters having different levels of sequence conservation. To understand how selection has acted, we quantified three important phenotypes: modal population expression level, phenotypic plasticity, and noise.

By comparing the variation in expression levels to the level of segregating genetic variation, we established that promoters with higher levels of genetic variation also have higher levels of phenotypic variation (**Fig. 3** and **Supplementary Figure 2**). This suggests that the decreased genetic variation observed in some promoters is due to stronger stabilizing selection.

To more directly measure the action of selection, we created a large number of random variants for each of the ten promoters. To create these we used MG1655 variants, which proved to be representative of segregating variants in the majority of cases. As the random variants were produced via PCR random mutagenesis, they had never been subject to selection. We first quantified the effects of these random unselected mutations on expression level. This comparison highlighted the necessity of using multiple environments to understand mutational effects, as we frequently observed environment-specific effects on expression (**Fig. 4**). Only a small number of studies have examined expression across multiple environments to understand promoter evolution (Duveau et al., 2017; Schaerli et al., 2018; Urchueguía et al., 2019); many more have used only a single environment (Duveau et al., 2018; Hodgins-Davis et al., 2019; Metzger et al., 2015; Schmiedel et al., 2019; Silander et al., 2012). However, bacteria often inhabit multiple different environments.

By comparing expression levels from segregating and random promoter variants, we showed that natural selection has acted such that segregating variants tend to have small-effect

413 mutations. However, the strength of the observed differences between the two groups varies
 414 among both promoters and environments. In general, promoters are more robust to mutations
 415 affecting expression level when expression is low or completely off (**Fig. 5**, *yhjX*, *mtr* or *dctA* in
 416 glucose). More specifically, we observed few strongly repressed promoters exhibiting increased
 417 expression due to random mutations.

418 Using a similar strategy to that above, we compared the phenotypic plasticity of segregating
 419 promoter variants and random promoter variants. Overall, we found that five out of ten random
 420 variants exhibited lower plasticity. In these cases, there may be stabilising selection on plasticity
 421 in natural populations. However, given that random variants generally exhibited lower plasticity,
 422 this leaves open the possibility that there is strong directional selection for regulation to be as
 423 plastic as possible, but that higher levels of plasticity are difficult to achieve as there is not
 424 sufficient genetic variation.

425 The random variants of *yhjX*, *lacZ*, *purA*, and *tpiA* did not exhibit different levels of plasticity
 426 than segregating variants. Interestingly, three out of four of these promoters are regulated solely
 427 via activation (*yhjX*) or repression (*purA* and *tpiA*). This contrasts with all the promoters for
 428 which random variants exhibited lower plasticity than segregating variants - all five are regulated
 429 by both activation and repression. It is possible that plastic responses from promoters with this
 430 simplified architecture are more robust to random mutations, while regulation from promoters
 431 having both repressors and activators is easily disrupted.

432 For *dctA*, we observed significantly higher plasticity for random variants compared to
 433 segregating variants in one pair of environments (**Fig. 6e**, pyruvic acid vs. L-malic acid, $p =$
 434 0.009). This result suggests that the *dctA* promoter is under selection to maintain the same
 435 expression level in both environments, and surprisingly, that this regulatory robustness is easily
 436 destroyed via very few mutations (**Fig. 6d**) - most random variants differ from the MG1655
 437 variant by only one or two mutations.

438 Finally, we have shown that cell-to-cell expression variability, or noise, is often subject to strong
 439 selection - as has been shown previously (Hornung et al., 2012; Metzger et al., 2015; Rossi et
 440 al., 2019; Schmiedel et al., 2019; Silander et al., 2012; Urchueguía et al., 2019). Importantly, the
 441 evidence we present here suggests that selection acts directly to decrease noise, rather than
 442 this being a correlated response to other selection pressures. In addition, we found that even
 443 single mutations have subtle but detectable effects on noise.

444 It is very possible that in cases in which we did not find significant changes in a phenotype
 445 between segregating and random variants it is possible that those promoters are not optimized
 446 for certain phenotypes in the environments considered. This might be true especially in cases
 447 when the bacteria do not encounter such environments very often.

448 We did not observe any strong correlations between the extent of segregating variation (**Fig. 1**)
 449 and the phenotypic consequences of *de novo* random mutations. For example, there was no
 450 evidence that random mutations generally had smaller phenotypic effects in highly polymorphic
 451 promoters. There are two possible explanations for this pattern. First, although our assays of
 452 expression phenotypes can detect differences between random and segregating mutations,
 453 there may be more subtle differences in mutational effects that we cannot detect. For example,
 454 there may be mutations with small effects that are detected by selection but not in this
 455 experimental context. The second possibility is that *de novo* mutations do have similar effects in
 456 highly polymorphic and conserved promoters, but selection is not as strong in highly
 457 polymorphic promoters, allowing through more small-effect mutations. This is supported by the
 458 correlation we observed between genetic variation and phenotypic variation in expression levels
 459 (**Fig. 3** and **Supplementary Figure 2**). To confirm this hypothesis fitness assays are required.

460 However, previous work suggests that fitness differences can be difficult to detect even when
 461 expression levels change by more than ten-fold (Keren et al., 2016). In the majority of results
 462 here, random mutations have less than two-fold effects on expression level. Thus, the approach
 463 we take here, comparing the phenotypes of segregating and random variants, may be a more

464 powerful method to infer the action of selection than assaying the effects of random mutations

465 on fitness relative to a single wild type.

466 Finally, we showed that taking into account all the regulatory behaviours we have quantified

467 here - expression level, phenotypic plasticity, and noise - that selection has acted such that

468 segregating SNPs have only minimal phenotypic effects. This conclusion is made more

469 compelling through the fact that the sequences of segregating promoter variants differ from

470 each other by, on average, more than three-fold compared to the sequences of random

471 variants. Thus, there are many single mutations that have large phenotypic effects - yet none of

472 these are segregating.

473 Methods

474 Promoter selection

475 We downloaded a database of transcription start sites (TSSs) driven by the σ^{70} factor from
 476 RegulonDB (Santos-Zavaleta et al., 2019). From this database, we filtered out TSSs denoted as
 477 “weak” in RegulonDB, or those outside of intergenic regions (IGRs). We also removed
 478 duplicated IGRs if multiple TSSs were present in the same IGR with the same orientation. This
 479 filtering resulted in 605 IGRs, each having one or more TSS, that were used for further analysis.
 480 Based on the TSS annotations, the IGRs together with 100 bp of flanking upstream and
 481 downstream sequences were extracted from a reference MG1655 genome using GenBank file
 482 annotation (Blattner et al., 1997). Throughout, we refer to IGRs together with the flanking
 483 sequences as “promoters”. The genomes of 135 environmental *E. coli* strains (Breckell &
 484 Silander, 2020; Ishii et al., 2006; Sakoparnig et al., 2021) were then blasted against the
 485 promoter sequences extracted from MG1655 with e-value cut-off set to 10^{-10} ; Blast version 2.7.1
 486 (Altschul et al., 1990). Blast hits not more than 100 bp shorter than the MG1655 reference
 487 sequence were extracted as segregating promoter variants from each of the 135 environmental
 488 *E. coli* strains. All segregating promoter variants of each promoter were then aligned via t-coffee
 489 version 11.0.8 with parameter mode procoffee (Notredame et al., 2000). We then calculated the
 490 average pairwise identity (API; t-coffee) and proportion of segregating sites (PSS) for each
 491 promoter and IGR (excluding the 100 bp flanking sequences).
 492 We also extracted open reading frames (ORFs) directly downstream of each of the 605 filtered
 493 promoters from the MG1655 reference genome (GenBank annotation). The same workflow for
 494 extracting segregating promoter variants was followed when extracting segregating ORF
 495 variants from the 135 strains of *E. coli* (changing the t-coffee parameter setting -type=dna). No
 496 extra flanking regions were included in the ORFs. Out of the 605 promoters originally identified

in the MG1655 genome, 429 were found in at least 130 of the 135 environmental *E. coli* isolates together with their downstream ORFs. For all further analyses and tests we used only these 429 promoters, IGRs, and ORFs.

To check whether any functional classes of ORFs were enriched for promoters with IGRs that have high or low sequence variation, we grouped the IGRs into the major categories defined by MultiFun (Serres & Riley, 2000) according to their downstream ORF. IGR variation (PSS and API) from each group was then compared to the rest of the groups together using a Wilcoxon rank-sum test. We also tested the correlation in sequence variation (PSS and API) between IGRs only and promoters (IGRs with 100 bp of upstream and downstream sequences flanking each IGR) using Spearman's correlation test. All the scripts used with the workflow described above can be found in the **Supplementary Information**.

From the resulting promoter dataset we selected ten promoters exhibiting various levels of sequence variation. We aimed at selecting promoters with obvious and easy differential regulation, i.e, either with known environments in which differential expression occurs or those whose annotated transcription factors suggested possible environments for differential expression. The selected promoters - *aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA* - differ more than ten-fold in their levels of genetic variation, while they all control expression of genes that are involved in bacterial metabolism or transport of material involved in the metabolism.

Promoter library construction

For each promoter (*aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA*) we created two libraries, one with segregating promoter variants and a second with randomly mutated promoter variants. The two promoter libraries were aliquoted into separate microplates, except for the *lacZ* promoter, which was aliquoted into a single microplate. Each microplate also contained the MG1655 variant of the promoter, a positive control consisting of the highly active murein

lipoprotein (*lpp*) promoter driving GFP expression, and a negative expression control consisting of a promoter-less pUA66 plasmid (Zaslaver et al., 2006). We describe the construction of each library type separately below.

525 Variants segregating in *E. coli* population

Both the vector pUA66 backbone (a low-copy number plasmid with a SC101 ori, a strong RBS, and GFPmut2) and segregating promoter variants were PCR amplified using Phusion High-Fidelity DNA polymerase with HF buffer (New England Biolabs). For promoter PCR amplification, 5µl of pooled DNA from strains with different variants of each promoter was used as a DNA template. The primers for promoter amplification contained 17 nucleotide overhangs which were homologous to the ends of the vector backbone for subsequent DNA assembly. All primers used in this study are listed in **Supplementary Table 1**.

For vector PCR amplification, 0.5 ng of plasmid DNA with pUA66 backbone served as a template. After confirming a successful PCR amplification of the products on 1% agarose gel, the template DNA was digested by DpnI from the remaining reaction volume (Li et al., 2011). The reactions were then column-purified and we assembled the PCR amplified vector and promoters using Gibson assembly (Gibson et al., 2009) with NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs). The assembly mix was then electroporated directly into the electrocompetent MG1655 strain. Transformed colonies which grew on LB agar plates with 50µg/ml Kanamycin were picked for Sanger sequencing across the insert in the vector backbone and stored as glycerol stocks. Clones with confirmed promoter variants matching the segregating ones were then grown in liquid LB with Kanamycin and used to create 96 well microplate glycerol stock libraries.

544 Random variants from PCR mutagenesis

545 We amplified the pUA66 backbone, DpnI treated, and column-purified it the same way as
 546 described for segregating promoter variants above. We produced the promoter inserts by
 547 performing error-prone PCR using the GeneMorph II Random Mutagenesis Kit (Agilent
 548 Technologies). We used the plasmid constructs with MG1655 promoter variants cloned into
 549 them as template DNA for the error-prone PCR aiming to achieve approximately 1.5 SNPs per
 550 promoter sequence. We used the same primers as for the segregating promoter variants
 551 (**Supplementary Table 1**). Each reaction with randomly mutated promoter variants was
 552 column-purified before Gibson assembly with the NEBuilder® HiFi DNA Assembly Master Mix
 553 (New England Biolabs). Each assembly mix was then electroporated into the MG1655 strain
 554 and colonies that grew on LB with Kanamycin were picked for Sanger sequencing and stored as
 555 glycerol stocks. Clones which had none or more than three SNPs in the cloned promoter insert
 556 were excluded as well as those with SNPs detected in the vector backbone (rare occasion). The
 557 rest of the strains were then re-grown in liquid LB with Kanamycin in 96 deep-well microplates
 558 overnight and 96 well microplate glycerol stock libraries were prepared from them.

559 Bacterial clones and environments

560 We performed all the promoter activity assays in the MG1655 genetic background of *E. coli*. All
 561 the clones had a pUA66 vector (Zaslaver et al., 2006) differing by promoter variants cloned
 562 upstream of the GFPmut2 gene (for details see *Promoter library construction* in **Methods**). We
 563 also included positive and negative expression controls which consisted of the highly active
 564 plpp::GFPmut2 strain from the Alon Zaslaver library collection and promoter-less pUA66 vector,
 565 respectively (Zaslaver et al., 2006).
 566 The assays were performed in 96 well microplates in M9 minimal media (Sigma-Aldrich)
 567 supplemented with MgSO₄, CaCl₂, a carbon source, and any additional reagents needed to

568 induce or repress expression from a particular promoter (**Table 2**). We grew all the clones in the
569 presence of 50µg/ml Kanamycin to prevent plasmid loss.

Promoter	Assay media	Incubation time prior flow cytometry	Environment abbreviation
<i>aldA</i>	0.4% glucose	4.5h	Glu
	0.4% fucose	4.5h	Fuc
	0.4% glycerol	4.5h	Gly
<i>yhjX</i>	0.4% glucose	4.5h	Glu
	0.4% pyruvic acid	4.5h	Pyr
	0.2% pyruvic acid	4.5h	Pyr2
<i>lacZ</i>	0.4% glucose	4.5h	Glu
	0.4% galactose	4.5h	Gal
	0.4% lactose	4.5h	Lac
<i>aceB</i>	0.4% glucose	4.5h	Glu
	0.4% pyruvic acid	4.5h	Pyr
	0.4% L-malic acid	7.0h	Mal
<i>mtr</i>	0.4% glucose	4.5h	Glu
	0.4% glucose + 1mM L-tryptophan	4.5h	Try
	0.4% glucose + 1mM L-phenylalanine	4.5h	Phe
<i>cdd</i>	0.4% glucose	4.5h	Glu
	0.4% glycerol + 2mM Cytidine	4.5h	Cyt
	0.4% glycerol + 3mM Adenosine monophosphate	4.5h	Amp
<i>dctA</i>	0.4% glucose	4.5h	Glu
	0.4% pyruvic acid	4.5h	Pyr
	0.4% L-malic acid	7.0h	Mal
<i>ptsG</i>	0.4% glucose	4.5h	Glu
	0.4% glycerol	4.5h	Gly
	0.4% D-mannose	4.5h	Man
<i>purA</i>	0.4% glucose	4.5h	Glu
	0.4% glycerol	4.5h	Gly
	0.4% L-arabinose	5.5h	Ara
<i>tpiA</i>	0.4% glucose	4.5h	Glu
	0.4% glycerol	4.5h	Gly
	0.4% L-arabinose	5.5h	Ara

Table 2: Promoter-environment combinations. All environments included M9 minimal salt media supplemented with MgSO₄, CaCl₂, and 50µg/ml Kanamycin.

Before introducing the clones into the assay media we first inoculated them into 0.5ml of M9 minimal media with 0.4% glucose and Kanamycin (M9 glucose) from a 96 well microplate glycerol stock using a 96-well pin replicator (Enzyscreen B.V.). We then incubated these microplates overnight at 37°C with shaking to revive the cells. After overnight growth in M9 glucose, we inoculated each grown clone into 0.5ml of one of the assay media in a 96 deep-well microplate using the pin replicator and incubated them for 24h at 37°C with shaking. After this second incubation we inoculated the clones into the same fresh assay media they grew in for 24h, but into three separate 96 deep-well microplates (to obtain triplicates for each clone). These cultures were incubated until they reached an exponential phase (**Table 2**).

Flow cytometry analysis

Once the cells reached exponential growth (**Table 2**) in the appropriate assay media, we diluted them into 1x PBS with ~2.5% formaldehyde and kept them on ice until performing flow cytometry analysis the same day. We performed the flow cytometry on a BD FACSCanto II machine using BD FACSDiva software version 6.1.3. We obtained the GFP fluorescence data through the 488 nm laser and a 513/17 nm bandpass filter. We set the number of events to record from each well to 20,000. We exported the acquired data from FACSDiva software into Flow Cytometry Standard files, and performed all cell gating and fluorescence analysis using custom R scripts (flowCore package version 2.0.1; see **Supplementary Information**). We gated cells based on their maximal kernel density of forward and side scatter values, using the ellipsoidGate function from the flowCore package, and keeping about 1/3 of all events. The modal population expression was calculated as the mean of the three maximal kernel density values from the GFP fluorescence signal of three replicates. The coefficient of variation (CV) was calculated separately for each of the three biological replicates (standard deviation divided by the modal population expression), and the mean of these values was used as the CV of the promoter. Replicates with fewer than 2,500 and 5,000 recorded events were excluded

595 from the calculation of modal population expression and coefficient of variation, respectively. We
 596 found that when fewer than 5,000 events were collected, a larger number of these were likely to
 597 be outlier events (e.g. machine noise), which affected the calculations of variance.
 598 When comparing segregating and random variants of the same promoter that came from two
 599 separate microplates, we obtained an offset for both modal population expression and
 600 coefficient of variation to minimise plate-effects. We calculated these offsets as the mean of the
 601 differences between the three controls present in each microplate, i.e., the MG1655 promoter
 602 variant, plpp::GFPmut2 (positive control), and pUA66 (promoterless negative control). All figures
 603 contain modal expression and coefficient of variation values that are corrected using these
 604 offsets. All scripts using the workflow described here, including the original data files can be
 605 found in the **Supplementary Information**.

606 Testing the correlation between segregating genotypic and phenotypic variation

607 To compare sequence promoter variation with variation in expression levels among segregating
 608 variants we used the promoter sequences (IGRs with 100 bp flanking regions) of the ten
 609 selected promoters (*aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA*). We measured
 610 modal population expression level in triplicates from each segregating promoter variant in three
 611 different environments (**Table 2**), and used the mean of these triplicate measures. We then
 612 calculated the standard deviation of modal population expression levels from all segregating
 613 variants of each promoter in each environment as a measure of phenotypic variation. For each
 614 promoter we thus had three values of segregating phenotypic variation (one for each
 615 environment). The MG1655 variant of the *mtr* promoter was excluded from the calculations due
 616 to a SNP in GFP causing lower fluorescence (**Supplementary Note**). We then used
 617 Spearman's correlation test to calculate the correlation between the phenotypic variation and
 618 genotypic variation using four metrics: PSS, API, total number of existing segregating variants

619 and number of cloned segregating variants (the code for these calculations can be found in the
620 **Supplementary Information**).

621 Mapping the effects of single SNPs to promoter sequence

622 When mapping the effect size and direction of randomly introduced SNPs we used modal
623 population expression values only from those random variants which had contained a single
624 SNP relative to the MG1655 promoter variant. Information about the TF binding sites, -10 and
625 -35 elements, and ORFs was taken from the EcoCyc database (Karp et al., 2018), and only the
626 annotations associated with σ^{70} driven TSSs were used. Using the Sanger sequencing results,
627 we identified the location of all SNPs for each random variant (the code for these calculations
628 can be found in the **Supplementary Information**).

629 Comparison of phenotypic variation between segregating and random variants

630 To test for differences in the variation in expression between the segregating and random
631 variants, we calculated the modal population expression values for each promoter variant in
632 both groups and all environments. We then tested for significant differences in variation in modal
633 population expression levels using the Fligner-Killeen test of homogeneity of variances. We
634 included values from the non-mutated MG1655 promoter variants in the sets with the
635 segregating variants, except for the *mtr* promoter due to a SNP in GFP (**Supplementary Note**;
636 the code for these calculations can be found in the **Supplementary Information**).

637 Comparing plasticity between segregating and random variants

638 We calculated the phenotypic plasticity of promoter variants across all three environments by
639 calculating the Euclidean distance of each datapoint in three dimensions to an isocline
640 representing null plasticity. The isocline is defined by equal values in the three dimensional
641 space, i.e., $x = y = z$. The three dimensions are defined by the modal population expression

values in the three environments specific for each promoter. Each datapoint (promoter variant) is thus defined by its expression values in all three environments. The closer a datapoint is to the isocline, the lower the plasticity.

To calculate plasticity in pairs of environments, we used an analogous method for two environments. To test whether natural selection had acted on plasticity (across all three environments and in pairs of environments) we compared plasticity values from all segregating (including MG1655 variants, except the one from *mtr* promoter - **Supplementary Note**) and all random variants for each of the ten promoters using the Wilcoxon rank-sum test (the code for these calculations can be found in the **Supplementary Information**).

Comparing noise between segregating and random variants

To determine the noise within an isogenic cell population of each promoter variant, we first excluded fluorescence values from the population that were lower or higher than three standard deviations from the modal population expression level. Then we calculated the coefficient of variation from this isogenic cell population as the standard deviation of the fluorescence divided by the modal population expression level. We next fitted a cubic smoothing spline (smoothing parameter $\lambda = 0.01$) to the modal population expression vs. the coefficient of variation values, using all (segregating and random) promoter variants. We determined the noise levels as the difference in measured coefficient of variation from the coefficient of variation predicted from the fitted spline. We then compared the noise values between segregating and random variants using Wilcoxon rank-sum test to determine whether selection had acted on noise (the code for these calculations can be found in the **Supplementary Information**). The MG1655 variant of the *mtr* promoter was excluded from the calculations due to a SNP in GFP causing lower fluorescence (**Supplementary Note**).

665 Comparing overall promoter activity between segregating and random variants

666 We calculated the mean and standard deviation for each promoter and phenotype (modal
667 population expression in all three environments, plasticity across all three environments and
668 transcriptional noise deviation for each of the three environments) using just segregating
669 variants. We then used these mean and standard deviation values of segregating variants to
670 calculate z-score for each individual promoter variant (both segregating and random) to
671 determine how much it differs in a particular phenotype from an average segregating variant. To
672 focus on the relative size of the change, rather than on directionality, we converted all z-scores
673 into absolute values. Giving all the phenotypes an equal weighting, we summed the z-scores for
674 each variant to measure its deviation from an average segregating variant in all the phenotypes
675 together. Due to a strong correlation between the z-scores for expression levels in the three
676 environments, we used only expression level z-scores from a single environment. This
677 environment was chosen so that the median expression from the segregating variants was
678 higher than 2.5 to remove environments causing promoter repression. If more than one
679 environment remained, we used the one least correlated with plasticity. This was done to
680 minimize non-independence of individual z-scores when calculating cumulative z-scores. We
681 then checked for statistically significant differences between the cumulative z-scores of
682 segregating (including MG1655 variants, except the one from *mtr* promoter - **Supplementary**
683 **Note**) and random variants using Wilcoxon rank-sum test (the code for these calculations can
684 be found in the **Supplementary Information**).

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691 Author contributions

692 MV and OKS conceived the project and designed the experiments and analyses. OKS
 693 supervised the project. MV performed all experiments and all analyses. MV wrote the paper with
 694 contributions from OKS.

695 Conflict of interests

696 The authors declare that there are no conflicts of interests.

697 Data availability

698 The original data files and scripts with data analyses that support the findings of this study are
 699 available in the **Supplementary Information** of this study.

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901 Supplementary Information

902 All scripts with access to original data files can be found at
 903 https://github.com/marketavlkova/2021_PromoterEvolution, the Supplementary Files can be
 904 accessed through Figshare DOI: <https://doi.org/10.6084/m9.figshare.c.5517228.v2>.

905 Supplementary Note

906 We found that the MG1655 *mtr* variant is expressed at lower levels than all other *mtr*
 907 segregating variants when grown in glucose with phenylalanine. In addition, the promoter
 908 variants generated via random mutagenesis of the MG1655 variant exhibited expression levels
 909 similar to the segregating variants rather than MG1655. We thus sequenced the plasmid
 910 containing the MG1655 promoter variant, and found that there was a single non-synonymous
 911 mutation in GFP at position 521 changing the codon GGA (G) to GAA (E). A small fraction of
 912 other constructs may also contain a mutation outside of the promoter region, but we expect that
 913 the large sample sizes we use will negate the effect of this small fraction on our conclusions.

Supplementary Figure 1: Polymorphisms in intergenic regions (IGRs) and open reading frames (ORFs) across 135 environmental isolates of *E. coli* and MG1655. **a)** The inverted average pairwise identity (100 - API) for IGRs and downstream ORFs varies by more than an order of magnitude. Each blue dot indicates an inverted API for an IGR-ORF pair when the IGR contains a transcriptional start site for the ORF. In red are IGRs that we selected for further study due to their different levels of sequence variation (*aldA*, *yhjX*, *lacZ*, *aceB*, *mtr*, *cdd*, *dctA*, *ptsG*, *purA*, and *tpiA*; see **Table 1**). **b)** The inverted API for IGRs differs little among different functional groups, as classified by the downstream ORF. CH denotes conserved-hypothetical. Unclassified, ORFs, and CH-ORFs all represent groups of ORFs with very limited information on function (Serres & Riley, 2000). Numbers next to each function group represent the p-value obtained via the Wilcoxon rank-sum test to test for differences in API in each group from all other inverted API values for IGRs outside of the group. Bold indicates significant p-values after the Bonferroni correction for multiple comparisons among functional groups. The black lines indicate the mean inverted API values of each functional group. **c)** and **d)** Correlation in sequence variation between IGRs and promoters (IGRs with 100 bp of flanking ORF regions). **c)** shows the proportion of segregating sites (PSS) and **d)** displays the inverted average pairwise identity (100 - API).

914

Supplementary Figure 2: Segregating genetic variation in promoters correlates with variation in expression levels. **a)** and **b)** Standard deviations in modal expression levels from segregating variants are correlated with the genetic variation of the promoter (IGR with 100 bp flanking regions). Panel **a** shows the correlation with API and panel **b** shows the correlation with the number of segregating promoter variants cloned and used for phenotypic assay. For each promoter, the standard deviation of modal population expression was measured in three environments (three dots per promoter, **Table 2**). The rho and p-values were calculated using Spearman's correlation test.

915

Supplementary Figure 3: Comparison of expression levels from promoters in pairs of environments. All x and y axes represent the modal population expression level in the particular environment written on the axis label. The blue dotted lines indicate equal expression levels in both environments, i.e., no phenotypic plasticity. The further from the line a promoter is, the higher the absolute difference is in expression from the promoter between the two environments (i.e., the higher its phenotypic plasticity).

916

Supplementary Figure 4: Fits of smoothing splines to modal population expression and coefficient of variation. A smoothing spline was fitted to all variants (segregating and mutagenized) in each environment. The term “noise” is used for the vertical deviation of each variant, i.e., deviation in the coefficient of variation from the fitted spline. The coefficient of variation is calculated as a standard deviation of log transformed expression levels (stdev) divided by modal population expression level (Mode).

Supplementary Figure 5: The lower sample sizes of random variants can increase p-values by two orders of magnitude. To test whether the not-significant p-value for *lacZ* promoter in plasticity (as in **Fig. 6c**) could be due to the lower number of random variants generated, we subsampled random variants of all other promoters to match the number of random *lacZ* variants (29 in total). We repeated this subsampling 1000-times, and for each iteration we compared the 29 subsampled random variants to all the segregating variants using Wilcoxon rank-sum test. This shows that the lower sample size for *lacZ* random variants likely decreased our power to find differences between segregating and random variants. The resulting p-values are plotted here. The horizontal red lines indicate p-values of 0.05, 0.01, 0.001, and 0.0001. The black lines indicate the p-values without subsampling (as in **Fig. 6c**).

918